REMARKS

Claims 1, 3, and 6-12 were pending in the application at the time the Office Action was mailed. Claims 7-10 were withdrawn from prosecution. Claims 1, 3, 6, 11 and 12 were rejected. By this amendment, claims 1, 7, 9 and 10 have been amended. Claim 8 has been canceled. No claims have been added. Therefore, claims 1, 3, 6, 11 and 12 are pending in the application. No new matter was added by virtue of these amendments and entry is respectfully requested.

Claim Rejections Under 35 U.S.C. 112, First Paragraph

Claims 1, 3, 6, 11, and 12 were rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description and enablement requirements. According to the Office Action, "[t]he disclosure does not teach a detection capability of 1 pg/µl *per se*, because 1 pg/µl is below the detection limits of the assay *unless* a 50 µl sample is used which would raise the level of NF-H in the sample to 50pg, the smallest amount that the assay can detect."

Claim 1 (from which claims 3, 6, 11 and 12 depend) has been amended herein to recite "wherein NF-H can be detected in quantities as low as 50 pg" instead of "wherein NF-H can be detected at a concentration of about 1 picogram/µl." Support for the amendment to claim 1 of "wherein NF-H can be detected in quantities as low as 50 pg" can be found, for example, on page 3, lines 29-31, and Figure 1.

Accordingly, withdrawal of these rejections is requested.

Claim Rejections Under 35 U.S.C. § 103

Claims 1, 3 and 6 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Hu et al. in view of Zemlan. The Office Action states:

> Hu (March 8, 2002) teaches a method of detecting neuronal injury in subjects with Alzheimer's disease (AD) and vascular dementia by using ELISA with antibodies that bind NF-H found in CSF samples taken from the subjects (abstract, Figures 2-3). Hu does not teach using blood samples. Zelman teaches a

method of detecting neuronal injury in subjects by using ELISA with antibodies that bind neurofilament proteins found in blood samples taken from the subjects (abstract and column 3, line 25 to column 4, line 42). It would have been obvious to one of ordinary skill in the art at the time of the invention to use the methods of Hu with the blood samples of Zelman because it is simpler and easier to procure a blood sample and assay it by ELISA than it is to procure a CSF sample by lumbar puncture because the blood sample can be simply taken from the arm (no usual side effects) while the CSF sample needs to be taken from the spinal cord region with the attendant risks of damaging the cord and then producing the side effects usually resulting from lumbar puncture such as headaches. The instant invention is *prima facie* obvious because the artisan would be motivated to take a simple venous blood sample (less time consuming than even a routine blood donation) than take a riskier CSF sample from the spinal cord region.

Claim 1 (from which all remaining claims depend) as amended herein recites a "method of detecting neuronal injury in a subject, the method comprising the steps of:

(a) providing a blood, serum, or plasma sample from the subject;

(b) contacting the blood, serum, or plasma sample with an antibody that specifically binds to NF-H in the sample;

(c) detecting the presence or amount of NF-H in the sample,

wherein NF-H can be detected in quantities as low as 50 pg; and

(d) correlating the presence or amount of NF-H in the sample with the neuronal injury."

Applicants respectfully assert that the combination of Hu et al. and Zemlan fails to render the claimed invention obvious for several reasons. First, the combination of references fails to teach or even suggest all claim limitations, e.g., "NF-H can be detected in quantities as low as 50 pg." Zemlan discloses an ELISA for tau (which is an entirely different protein unrelated to NF-H) having a sensitivity of 30 μg per well when assaying CSF samples for tau. This level of sensitivity is *several* orders of magnitude less sensitive than the method and assay of the claimed

invention and applies to an entirely different protein (tau) than that recited in claim 1 (NF-H). Clearly, Zemlan is not an enabling reference with regard to a method of detecting neuronal injury in a subject including detecting the presence or amount of NF-H in a blood, serum, or plasma sample having a sensitivity as low as 50 pg (as recited in claim 1 as amended herein). Combining this nonenabling reference with Hu et al. would not result in the claimed invention. Furthermore, there is no teaching in either of these references that would suggest how to or motivate one to alter the teachings of these references to result in the claimed invention. Hu et al. also does not even mention assaying blood, serum or plasma samples (as recited in claim 1). Instead, Hu et al. teaches that a rather unrefined ELISA method using samples from lumbar CSF reveals differences in the "relative level" of NF-H as a function of normal aging, Alzheimer's disease and some other neurological states. The assay described in Hu et al. does not even address the actual amounts of NF-H present even in CSF samples. Importantly, Hu et al. neither teaches nor suggests that NF-H, in response to a neuronal injury, Alzheimer's disease or any other state, can be found in blood, plasma, or serum.

It is widely recognized that CSF is vastly easier to work with than blood, plasma or serum, which contain far more protein and are present at much higher volumes in the body. Accordingly, any brain injury biomarker is greatly diluted on entry to blood, and becomes a much smaller part of a much larger and complex whole. As a result, many biomarker proteins which can be easily detected in CSF have not been reported to be detectable in blood, plasma or serum. This may be due to one or a combination of several reasons. Possible such biomarker proteins simply never get past the blood brain barrier, or they are degraded, modified or sequestered in one of several possible ways on entry into blood. Clearly any of these problems could have rendered the claimed invention unworkable. In addition, detection is absolutely dependent on an assay of sufficient sensitivity and avidity which can detect small amounts of protein against the large background of irrelevant blood proteins. Failure to develop such an assay would also render the invention unworkable. In addition, NF-H, with an SDS-PAGE molecular weight of 200kDa, is a much larger protein than tau (30kDa), and is also normally present as at least a multimer, while tau is monomeric. Furthermore, by far the majority of NF-H molecule is present in the phosphorylated, axonal form, which from the teachings of the prior art,

is known to be particularly resistant to proteases (Pant, Biochem. J. 256:665-8 1988, Greenwood et al. J. Neurochem. 61:191-199 1993). All these factors make it *a priori* less likely (and less obvious) that NF-H would be a viable brain injury and degeneration marker comparable to or (as appears to be the case) superior to tau. Other brain injury and degeneration biomarkers studied to date are either smaller than tau i.e (S100β, 11kDa, Myelin basic protein, 18kDa) or not much bigger (neuron specific enolase and glial fibrillary acidic protein ~50kDa). At the time the application was filed, the conventional wisdom in this area was that good novel biomarkers were likely to be relatively small proteins which would have fewer problems diffusing away from injury sites and crossing the blood brain barrier.

The combination of Hu et al. and Zemlan also fails to render the claimed invention obvious because the claimed invention was not "obvious to succeed" as asserted by the examiner on page 6 of the Office Action and yielded unexpected results, i.e. a method of detecting neuronal injury by detecting or measuring NF-H in a blood, serum or plasma sample at quantities as low as 50pg. Applicants' development of the methods and reagents claimed in the present application was not at all straightforward, but instead required considerable expertise in protein purification, novel antibody development, antibody characterization coupled with ELISA development, and optimization which required considerable further experimentation. Given the non-obvious finding that NF-H does in fact enter the blood following a variety of damage and disease states in measurable amounts, Applicants' development of an assay capable of accurately measuring low NF-H levels in the complex background of blood was not at all straight forward.

The 1.132 Declaration by Applicant Dr. Gerry Shaw, filed herewith, describes the extensive experimentation that was involved to result in the claimed invention, the potential problems that could have prevented development of the claimed invention, and the unexpected results yielded by the claimed invention. One of skill in the art combining the teachings of Hu et al. and Zemlan would not have a reasonable expectation of success of yielding an assay for detecting NF-H in blood, serum or plasma samples having a sensitivity as low as 50 pg due to the unpredictability of detecting NF-H in blood, serum or plasma samples at such low concentrations and because Zemlan is a nonenabling reference. Zemlan includes *no* data regarding neurofilament proteins – from any bodily fluid. Zemlan describes experiments

involving only tau proteins. As mentioned above, tau and NF-H are *entirely different* proteins. Tau and neurofilament subunits each belong to two distinct and ancient protein families with quite different domain organizations, functions, binding properties, gene structures and apparent evolutionary origin. Tau belongs to the microtubule associated protein family, which is distinct from the intermediate filament protein family to which NF-H belongs not only in humans, but also in *C. elegans* and all more primitive multicellular animals studied to date. Zemlan provides no guidance whatsoever as to which antibodies could be used to detect NF-H (or any other neurofilament proteins) in either a CSF or a blood, serum, or plasma sample, and provides no experimental evidence whatsoever that NF-H (or any other neurofilament proteins) can be detected in either CSF or blood, serum or plasma under any circumstances. Applicant respectfully submits that the "working example" the examiner refers to on page 5 of the Office Action is not germane to the claimed invention, as this "working example" involved experimentation only with tau protein – not NF-H protein, which is an *entirely different and unrelated* protein with quite different size, function, multimerization, solubility, binding properties and evolutionary origin.

In further support of nonobviousness, Applicants provide evidence of commercial success in the 1.132 Declaration filed herewith. Despite no significant effort at advertising, ELISA kits which are encompassed by the claimed invention have generated ~$40,000 in sales to date. In addition, Applicants are licensing the claimed invention to two major biotechnology companies, namely Millipore and BioVendor, with several other companies being interested or in negotiations. Applicants have published five peer reviewed research reports based on this application, and he alone or with collaborations has obtained seven different extramural grants to investigate the utility of this application in a variety of damage and disease states. Five other grant proposals and two further research reports are under review, and collaborations with more than twenty researchers located all over the world are underway. In addition, there have been several unsolicited articles in the popular and scientific press describing the claimed invention. In summary, there can be no question that the invention has generated considerable interest from federal and foundation grant agencies, from the biotechnology industry, from scientists and clinicians and from the popular and biotechnology press. It is clear that the claimed invention

provides an approach of great potential interest and utility, which required a specific expertise and considerable experimentation to bring to fruition, and it is difficult to see how this was possibly obvious or straightforward over the cited references.

Based on the foregoing, Applicants submit that the cited references do not render the present invention obvious within the meaning of 35 U.S.C. 103. Applicants submit that neither of the cited references, nor the combination thereof, teach or suggest all the limitations of the claims as amended herein, nor do the references suggest modifying their teachings to arrive at applicant's invention. Applicants further submit that even if one was motivated to combine and modify the teachings of Hu et al. and Zemlan to result in the claimed invention, one would have no reasonable expectation of success of yielding an assay for detecting neuronal injury in a subject including the step of detecting NF-H in a blood, serum, or plasma sample in quantities as low as 50 pg. In addition, the Zemlan reference is a nonenabling reference, as it describes absolutely no experiments performed involving NF-H. Furthermore, the claimed invention yielded unexpected results, and Applicants provide evidence of commercial success of the claimed invention in the 1.132 Declaration filed herewith.

In view thereof, Applicants respectfully request reconsideration and withdrawal of the instant rejection.

Claims 1, 3, 6 and 11 were rejected under 35 U.S.C. 103(a) as being unpatentable over Hu et al. in view of Zemlan and further in view of Grainger et al. (US Patent No. 5,595,722). According to the Office Action, "Grainger discloses that chicken polyclonal antibodies are suitable to be used in ELISAs to routinely detect and assay proteins such as TGF-β in serum and plasma samples" and "the use of chicken polyclonal antibodies in combination with the previously applied art would have yielded predictable results to one of ordinary skill in the art at the time of the invention."

Applicants respectfully disagree with this assertion because the combination of Hu et al. and Zemlan does not render the claimed invention obvious for the reasons set forth above, and the addition of Grainger et al. also fails to render the claimed invention obvious. Grainger et al. discloses using an ELISA to detect TGF-β in serum and plasma samples. TGF-β is a protein

found in blood, plasma and serum samples as part of a normal signaling function, so detecting it there with an appropriate antibody is not surprising and even when combined with the teachings of Hu et al. and Zemlan, provides absolutely no guidance for detecting NF-H in a blood, plasma or serum sample in quantities as low as 50 pg for detecting neuronal injury. In contrast to and as taught by Applicants in the present application, NF-H is not found normally at detectable levels in blood, plasma or serum. As shown by the Applicants, it is, however, present at detectable levels following various kinds of CNS injury or disease. Since it is normally a cytoplasmic protein of axons, the presence of this protein in blood, plasma or serum unambiguously indicates recent neuronal damage or degeneration, as described in the present application. The teachings of Grainger et al., do not even mention NF-H or neurofilament protein, and when combined with Hu et al. and Zemlan, fail to teach or suggest all claim limitations, and fail to provide any guidance to one of skill in the art how to detect neuronal injury in a subject by detecting NF-H in a blood, serum, or plasma sample in quantities as low as 50 pg.

Based on the foregoing, Applicants submit that the combination of cited references does not render the present invention obvious within the meaning of 35 U.S.C. 103. The combination of references fails to teach all claim limitations, and also includes no motivation or suggestion to modify the references such that combining them would result in the claimed invention. In addition, Zemlan is a nonenabling reference. Applicants' discovery and development of the claimed invention was therefore not straightforward, yielded unexpected results, and has resulted in commercial success.

Withdrawal of this rejection is therefore respectfully requested.

Claims 1, 3, 6 and 12 were rejected under 35 U.S.C. 103(a) as being unpatentable over Hu et al in view of Zemlan and further in view of Posmantur et al. According to the Office Action, "it would be obvious to try to look for the missing NF-H protein from the brain in the blood within a few hours of a neuronal injury as taught by Postmantur with the methods disclosed by Hu and Zelman, and the combination with the previously applied would have yielded predictable results to one of ordinary skill in the art at the time of the invention, absent evidence to the contrary."

Applicants respectfully disagree with this assertion on several grounds. First, because NF-H levels in the brain may begin to decrease as soon as 3 hours post-injury (as taught by Posmantur et al.) does not mean that the "missing NF-H protein from the brain" would necessarily be found in the blood and that it would therefore be obvious for one to look for this missing protein in the blood within a few hours of a neuronal injury. The injury model used by Posmantur et al. is a contusion, providing a mechanical shock but no marked cutting of tissue, so that release of significant amounts of neuronal protein would not be expected, resulting in most of the loss of NF-H 3-24 hours post-injury being due to proteolysis within damaged neurons, rather then release of NF-H. In fact, the focus of Posmantur et al. is clearly on the degradation of NF-H *in the cytoplasm* of damaged neurons and the partial amelioration of this degradation by inhibitors of calpain proteases. As recited in Posmantur et al., with italics for emphasis, "[d]egradation of cytoskeletal proteins including neurofilaments, MAP2, and spectrin, all of which are preferred substrates for calpain, and the appearance of putative calpain breakdown products to neurofilaments and spectrin strongly implicate the involvement of the *intracellular* cysteine protease calpain in experimental brain injury. Two known isoenzymes of calpain exist in the CNS. Calpain 2 has a millimolar sensitivity to calcium and primarily located *in axons*. In contrast calpain 1 is located in *neuronal somata, dendrites, axons and glia*." Posmantur et al. examined NF-H *remaining* after injury and the major finding was that more NF-H remained following inhibition of one of the several proteases potentially involved in NF-H degradation. Possibly if all NF-H proteases were inhibited, there would have been little or no loss of NF-H over the 24 hour time period studied, indicating that *none* of the reduction in the level of brain NF-H seen was due to release into blood. In addition, the assay used by Posmantur et al. measured the amount of the intact NF-H protein band. Cleavage of this band does not necessarily mean that NF-H is no longer in the brain, since no data is presented on what happened to the fragments generated by cleavage of the NF-H band. Other mechanisms by which NF-H levels could decline following injury are nowhere addressed in Posmantur et al., although there are several apart from release into the blood. For example, intact bundles of neurofilaments within axon segments or free in the extracellular space could be endocytosed by microglia or other phagocytic cells. The reduced level of NF-H protein could be due to reduced synthesis, known

to occur after neuronal injury. The neurofilament proteins could be released from damaged neurons into the CSF but never reach the blood for any one or a combination of several reasons. They may become degraded in the CSF or they may not be able to cross the blood brain barrier- even today there is little data on the overall integrity of the blood brain barrier following a contusion injury, and the diffusion barrier is likely to be unpredictably different for proteins of different sizes and properties. Neurofilament subunits could enter the blood stream but become rapidly degraded, modified or sequestered by one of several possible mechanisms. Finally, NF-H protein is heavily concentrated in large diameter projection axons, which are a minor component of the cortical regions damaged in the Posmantur et al. experiments.

Furthermore, even if the combination of Zemlan, Hu et al. and Posmantur et al., would have led one skilled in the art to postulate that NF-H might enter the blood stream in measurable amounts, combining the teachings of these references would not have resulted in the claimed invention. At the time the present application was filed, Applicants alone developed an NF-H ELISA which can detect NF-H in blood samples in quantities as low as 50pg and which is described in the present application and encompassed by the claims as amended herein. As described above, Applicants' development of the claimed invention required considerable experimentation and particular expertise, yielded unexpected results and resulting in commercial success.

In summary, Posmantur et al. does not suggest to one of skill in the art to analyze a blood sample within a few hours of a neuronal injury for NF-H protein. Combining the teachings of Posmantur et al. with Zemlan and Hu et al. would not result in the claimed invention for several reasons. The combination of Hu et al. and Zemlan does not render the claimed invention obvious for the reasons set forth above, and the teachings of Posmantur et al. combined with Hu et al. and Zemlan also fail to provide a *prima facie* case of obviousness. The combination of Hu et al., Zemlan and Postmantur et al. fails to teach or suggest all claim limitations, and fails to provide any guidance to one of skill in the art how to detect neuronal injury in a subject by detecting NF-H in a blood, serum, or plasma sample in quantities as low as 50 pg. Applicants' discovery and development of the claimed invention was not straightforward, and has resulted in commercial success.

Withdrawal of this rejection is therefore respectfully requested.

CONCLUSION

The currently pending claims before the examiner are supported throughout the specification and are patentable over the prior art. No new matter has been added. This application is now in full condition for allowance, and such action is respectfully requested.

The Commissioner for Patents and Trademarks is hereby authorized to charge the required fee for a retroactive extension of time, and any underpayment of fees or credit any overpayment of fees to Deposit Account No. 50-0951.

The examiner is cordially invited to call the undersigned if clarification is needed on any matter within this amendment, or if the examiner believes a telephone interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,
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